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Decomposition of Nitroguanidine

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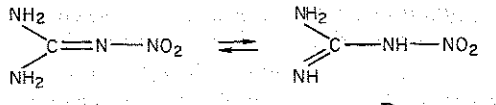
■ Nitroguanidine was not susceptible to aerobic biodegradation in activated sludge, and it was stable under sterile reducing conditions. Nitroguanidine was cometabolized by anaerobic sludge microorganisms to nitrosoguanidine after acclimation. There was no further microbial reduction of nitrosoguanidine (no aminoguanidine, hydrazine or urea was detected in culture extracts). Nitrosoguanidine decomposed nonbiologically and formed cyanamide, cyanoguanidine, melamine, and guanidine. All products were identified by thin-layer chromatography and mass spectroscopy. A pathway for the degradation of nitroguanidine is proposed. No ammeline, ammelide, or cyanuric acid was detected. Nitroguanidine and nitrosoguanidine were sensitive to UV light.

Introduction

Nitroguanidine is used as a component of military propellants. It is water soluble, and quantities may enter the environment via discharge streams from handling facilities (*1*, *2*). Insufficient information is available in the literature on the biological fate of nitroguanidine to assess environmental concerns.

Nitroguanidine is a nitroimino compound that exists in two tautomeric forms. Form A predominates in acidic, neutral or slightly basic media (*3*).



(1)

A B

The purpose of this investigation was to evaluate the susceptibility of nitroguanidine to microbial degradation. A further object was to gain insight into intermediate products formed during the decomposition process as well as evaluate the potential hazards associated with these compounds.

Experimental Methods

Media. Basal salts contained 1.0 g of K_2HPO_4, 1.0 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2$, and 0.01 g of NaCl per liter of distilled water adjusted to pH 7.0. $NH_4H_2PO_4$ (2.0 g/L) and glucose (1.0 g/L) were added as indicated. Nutrient broth concentrations ranged from 0.8 to 8.0 g/L.

Culture Conditions. Aerobic batch cultures were incubated in 250-mL Erlenmeyer flasks each containing 100 mL of media at 30 °C on a New Brunswick G24 environmental incubator shaker. Anaerobic (unaerated) batch cultures and sterile controls were incubated at 37 °C in 250-mL Erlenmeyer flasks filled with media. Some anaerobic controls and incubations contained 0.05% DL-dithiothreitol as a reducing agent.

New Brunswick Bio Flo Model C30 bench-top chemostats for continuous culture were maintained under aerobic and anaerobic conditions. The media used in aerobic chemostats were either basal salts with nitrogen and glucose or nutrient broth (2 and 4 g/L). Retention time was 7 days; the temperature was maintained at 30 °C, and the influent nitroguanidine concentration ranged from 75 to 100 ppm (μg/mL).

Anaerobic chemostats were run with nutrient broth (2, 4, and 8 g/L), basal salts, basal salts with glucose, and basal salts with glucose and nitrogen. Initial retention time was 7 days and later dropped to 4 and 2 days. Nitroguanidine influent concentrations ranged from 50 to 100 ppm.

Chemostats were operated continuously for up to 3 months at 37 °C.

Aerobic cultures were inoculated with activated sludge from the Marlborough Easterly sewage treatment plant (Marlborough, MA) and anaerobic cultures with digest from the Nut Island sewage treatment plant (Boston, MA). The aerobic and anaerobic sludges contained <0.1% and 1.7% total solids, respectively. Sludge samples were diluted 100-fold with 0.85% KCl and filtered, and 0.5 mL was added to the culture flasks.

Chemicals. Nitroguanidine was obtained form Radford Army Ammunition Plant, Radford, VA and recrystallized. Nitrosoguanidine was prepared according to Davis and Rosenquist (*4*). Ammeline was prepared according to Cloak (*5*) and ammelide according to Diebner (*6*). Aminoguanidine, cyanamide, cyanoguanidine, cyanuric acid, guanidine hydrochloride, and melamine were purchased from Eastman Kodak, Co., Rochester, NY. Hydrazine dihydrochloride and urea were purchased from Fisher Scientific Co., Medford, MA.

Mutagenicity Testing. The Ames screening test for mutagenicity was performed with nitroguanidine and nitrosoguanidine according to standard procedures (*7*, *8*). Five strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537, and TA1538) were used to test concentrations from 5 to 5000 μg of each compound per plate with and without metabolic activation.

High-Performance Liquid Chromatography (HPLC). Nitroguanidine and nitrosoguanidine concentrations were determined on a DuPont 830 liquid chromatograph with a Perkin-Elmer LC55 variable-wavelength detector at 263 nm and a Columbia Scientific Supergrator-2 programmable computing integrator. The mobile phase was methanol/water (10/90). Culture media samples were clarified by centrifugation for 10 min at 12000 rpm and filtered through 0.2-μm filters. Injections of 10 μL were made onto a 25 cm × 4.6 mm DuPont Zorbax ODS reverse-phase column at 35 °C and 1200 psi (8.273 × 10^3 kPa). Retention times were 2.8 and 2.5 min, and detection limits were 100/ppb (ng/mL) and 500 ppb for nitroguanidine and nitrosoguanidine, respectively.

Isolation of Nitrosoguanidine. Nitrosoguanidine was isolated from 4 L of spent media from an anaerobic chemostat that had been operated at a 7-day retention time with an influent containing 4 g/L of nutrient broth and 75 ppm of nitroguanidine. No nitroguanidine was detectable in the spent media, and nitrosoguanidine was present at 25 ppm. The medium was centrifuged at about 5000*g* for 10 min with a Sorvall SS-34 rotor, and the supernatant was filtered through a 0.45 μm filter and evaporated to dryness at 50 °C on a rotary evaporator at 15 mmHg (1.999 × 10^3 Pa). The dry residues was resuspended in warm methanol and used for analysis by thin-layer chromatography (TLC), HPLC, and mass spectroscopy (MS). TLC was performed by using ethanol/water (50/50) as the developing solvent on cellulose-coated plates with a fluorescent indicator. Spots were visualized under UV light and with iodine vapors. MS was performed in the electron impact (EI) and chemical ionization (CI) modes on a Finnigan Model 4000 mass spectrometer by probe analysis. Attempts were made to acetylate and silylate nitrosoguanidine for gas chromatographic MS analysis. These reaction mixtures were chromatographed on a capillary column, DB1, 25 m × 0.32 mm i.d., FS (J&W Scientific, Orangevale, CA) with a temperature program from 100 to 300 °C over 15 min.

Isolation of Cyanamide, Cyanoguanidine, Guanidine, and Urea. A 3-L sample of spent media from anaerobic chemostat cultures was centrifuged, filtered, and rotoevaporated as before. The concentrate was extracted with warm methanol and cochromatographed with standards on cellulose TLC media. The chromatograms were developed with 1-butanol/ethyl acetate/water (4/1/1) and visualized with alkaline ferricyanide/nitroprusside spray (*9*). Cyanamide (R_f 0.78) and cyanoguanidine (R_f 0.53) were purple, guanidine (R_f 0.26) was orange, and urea (R_f 0.35) was red orange. The media concentrate was also extracted with ammonium hydroxide and chromatographed in the same manner. Unknown spots of interest were scraped off TLC plates and eluted, and their identities were confirmed by MS. Other attempts were made to detect urea as the xanthydrol derivative (*10*).

Isolation of Melamine, Ammeline, Ammelide, and Cyanuric acid. Concentrates from spent nitroguanidine cultures were prepared as before. The residues were extracted with water, methanol, ammonium hydroxide, or formic acid in different experiments. The extracts were subjected to TLC on cellulose media without fluorescent indicator. The chromatograms were developed with 3 N ammonium hydroxide/methanol (60/75) (*11*). Visualization was carried out with silver nitrate spray followed by heating at 100 °C and with *p*-(dimethylamino)benzaldehyde (*9*). Media extracts were partly cleaned by passage through C-18 SEP-PAK cartridges (Waters' Associates, Milford, MA). Water and methanol washes were chromatographed on cellulose media which were also developed in 1-butanol/ethyl acetate/water (4/1/1). Standards were chromatographed with unknowns, and appropriate spots from the unknowns were scraped off and eluted for MS analysis. In 1-butanol/ethyl acetate/water, melamine had R_f 0.38 and appeared yellow after spraying with *p*-(dimethylamino)benzaldehyde. In ammonium hydroxide/methanol (60/75) the R_f was 0.78.

Attempted Detection of Aminoguanidine. Nitroguanidine culture media were examined for the presence of aminoguanidine by HPLC which was performed as with nitroguanidine. The following parameters were used: detection at 210 nm, pressure at 1000 psi (6.894 × 10^3 kPa), solvent composition at 15/85, methanol/water, and the column at room temperature. The retention time for aminoguanidine was 2.2 min, and it emerged just ahead of nitrosoguanidine and nitroguanidine. The limit of detection was 500 ppb.

Attempts were also made to identify aminoguanidine from the biological systems as a salicylaldehyde derivative. An aminoguanidine standard was derivatized, and the benzene extracts from this reaction and the derivative from the culture were chromatographed on Eastman silica gel TLC medium with fluorescent indicator, with isopropanol as developing solvent. Visualization of the chromatogram by a UV lamp showed that no detectable amount of aminoguanidine was present in the culture extract.

Attempted Isolation of Hydrazine as Salicylazine. The derivatized culture extracts obtained in the preceding section were cochromatographed with salicylazine in the same manner with benzene as developing solvent. Absence of a spot corresponding to salicylazine showed that a detectable quantity of hydrazine was not present in the culture medium.

In an attempt to form derivatives prior to concentration, a 1.5-L portion of the clarified culture medium was derivatized in situ by addition of 0.25 g of salicylaldehyde, stirring overnight at room temperature, and extraction with benzene. Neither aminoguanidine nor hydrazine could be detected by cochromatography with the salicylaldehyde derivatives as before. No salicyaldehyde derivative from

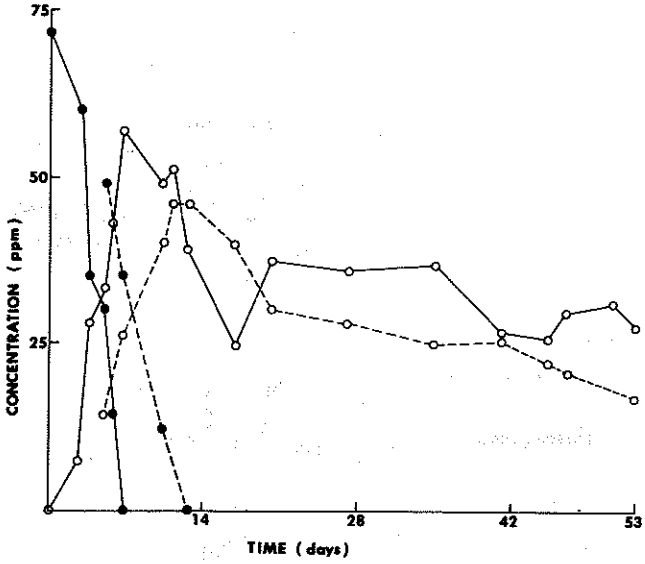


Figure 1. Biotransformation of nitroguanidine under anaerobic conditions in continuous culture: nitroguanidine (●), nitrosoguanidine (O), culture vessel (—), product reservoir (---).

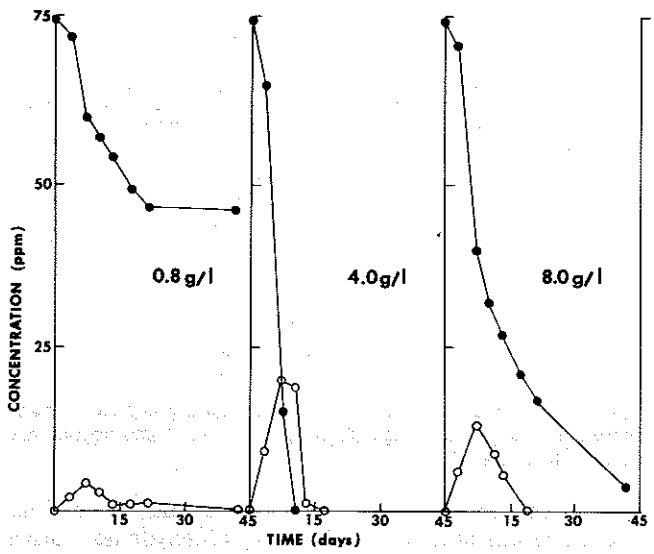


Figure 2. Biotransformation of nitroguanidine under anaerobic batch conditions in nutrient broth of different strengths: nitroguanidine (●), nitrosoguanidine (O).

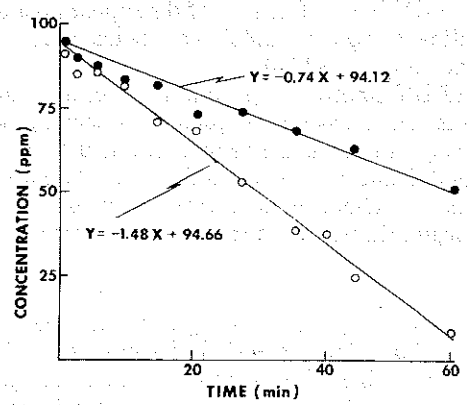


Figure 3. Sensitivity of nitroguanidine and nitrosoguanidine to shortwave UV light: nitroguanidine (●), nitrosoguanidine (O).

hydrazine or aminoguanidine could be detected by MS in any of the extracts.

UV Sensitivity of Nitrosoguanidine and Nitroguanidine. Solutions of nitrosoguanidine and nitroguanidine in distilled water at pH 6.0, 100 ppm in concentration and 2.5 cm deep, were stirred in beakers under a germicidal UV lamp (Sylvania G15T8). Samples were withdrawn periodically, and nitroguanidine and nitrosoguanidine concentrations were determined by HPLC.

Nitrosoguanidine was also irradiated in situ on cellulose TLC media (with fluorescent indicator). The media were exposed until the nitrosoguanidine had partially disappeared (10 min). The chromatograms were developed in 1-butanol/ethyl acetate/water (4/1/1) and visualized with alkaline ferricyanide or ninhydrin sprays.

Results

Under aerobic conditions no decomposition of nitroguanidine was observed in either batch or continuous cultures. During the first 3 weeks of incubation there was no significant degradation of nitroguanidine in anaerobic continuous cultures in 4 g/L of nutrient broth. Significantly enhanced rates of disappearance of nitroguanidine were detected in both continuous cultures (Figure 1) and batch cultures (Figure 2) after subculturing three times for new anaerobic incubations using the adapted cells. Figure 1 shows the rate of biotransformation of nitroguanidine (initial concentration 75 ppm) in anaerobic continuous culture (4 g/L of nutrient broth at a 7-day retention time). Within 7 days no nitroguanidine was detected in the culture vessel, and within 12 days there was none in the product reservoir. During this time nitrosoguanidine accumulated in the medium. The concentration of nitrosoguanidine rose to 60 ppm in the culture vessel by day 7 and leveled off between 25 and 40 ppm over a 53-day period. In the spent medium (product reservoir) the concentration of nitrosoguanidine rose to a maximum of 45 ppm by day 12, and then decreased slowly to slightly below the concentration in the culture vessel. Extended periods of continuous culture did not result in lower levels of nitrosoguanidine.

Alterations in media composition (2 or 4 g/L of nutrient broth) or shorter retention times (2 or 4 days) only resulted in slower rates of disappearance of both nitroguanidine and nitrosoguanidine. No decomposition occurred in cultures without nutrient broth (basal salts, basal salts with nitrogen, and basal salts with nitrogen and glucose). There were no conditions found in which the nitrosoguanidine level fell below about 20 ppm in the continuous culture vessel.

Complete disappearance of nitroguanidine and nitrosoguanidine in batch cultures after subculturing could be achieved, but only in a narrow range of media composition (Figure 2). In 4 g/L of nutrient broth both compounds had completely disappeared by day 17, while in 0.8 and 8 g/L of nutrient broth incomplete degradiation of nitroguanidine took place.

The identity of the nitrosoguanidine isolated from anaerobic continuous cultures was established by comparative TLC and HPLC with a standard sample. The mass spectrum of nitrosoguanidine with use of the probe did not provide unequivocal confirmation of structure due to the production of a large number of low molecular weight fragments. Only at very high concentrations could any parent ion be detected at m/z 88 in the EI mode or m/z 89 in the CI mode. No evidence was found for the formation of the acetylated or silyated derivative of nitrosoguanidine.

Cyanamide, cyanoguanidine, melamine, and guanidine were identified in extracts of culture media concentrates both by TLC and GC/MS. No urea was detected by TLC or as a xanthydrol derivative. No ammeline, ammelide, or cyanuric acid was detected in culture extracts by TLC and MS. No aminoguanidine was detected by MS, HPLC,

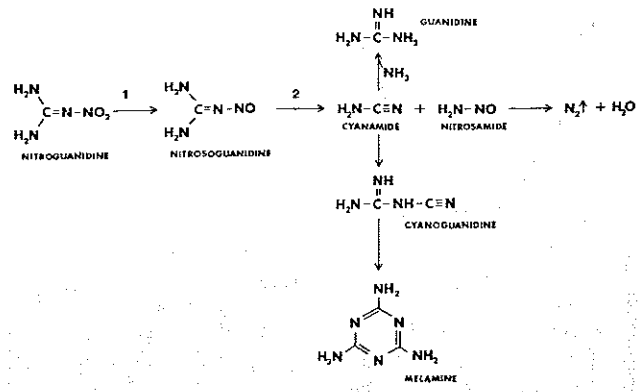


Figure 4. Scheme for chemical and biological degradation of nitroguanidine. Step 1 is biologically mediated; step 2 and beyond are chemically mediated.

or as a salicylaldehyde derivative by TLC and GC, and no hydrazine was detected as the salicylaldehyde derivative by TLC and GC.

Figure 3 illustrates the rates of disappearance of nitroguanidine and nitrosoguanidine in aqueous solutions under shortwave UV light. The rate of disappearance of nitrosoguanidine (slope = −1.48) was twice that of nitroguanidine (slope = −0.74).

TLC of UV photolysis products indicated that cyanamide was formed as nitrosoguanidine decomposed. No evidence of urea or guanidine was found, and the reaction products did not react with ninhydrin. Apparently the photolytic and chemical pathways for the decomposition of nitrosoguanidine result in similar products.

Discussion

Nitroguanidine did not biodegrade until the anaerobic cultures had a sufficient period of acclimation. Once acclimated, anaerobic cultures readily reduced nitroguanidine cometabolically. This transformation did not occur under aerobic conditions. No evidence was found for further microbiological reduction of nitrosoguanidine. Unlike nitroguanidine, we were unable to acclimate cells to completely metabolize nitrosoguanidine by further reduction. Probable reduction products would be hydrazine and urea or aminoguanidine and guanidine. Hydrazine, urea, and aminoguanidine were never detected during these studies, and guanidine, although present in trace amounts, can be postulated to arise by an alternative pathway.

It is probable that under these conditions nitrosoguanidine decomposes nonbiologically. Significant quantities of cyanamide, cyanoguanidine, and melamine were identified in the culture effluents. These compounds were reported to be among the products of the chemical decomposition of nitrosoguanidine (*4*). The most likely sequence of reactions can be diagrammed as in Figure 4. Nitrosoguanidine decomposes to cyanamide and nitrosamide. The cyanamide polymerizes to its dimer, cyanoguanidine, and finally to its trimer, cyclic melamine. Cyanamide can also react with ammonia to form guanidine. The nitrosamide formed is transitory and decomposes to nitrogen gas and water. Under the conditions prevailing in these experiments, these reactions apparently proceed relatively slowly as all intermediates with the exception of the unstable nitrosamide were successfully purified and identified by TLC and GC/MS.

In sterile nutrient broth batch culture controls, under anaerobic conditions with a reducing agent present, there was no change in the concentration of nitroguanidine, and there was no evidence for nonbiological reduction to nitrosoguanidine. Sterile nutrient broth controls with nitrosoguanidine revealed similar patterns of substrate disappearance and product formation, supporting the notion that the decomposition steps from nitrosoguanidine are nonbiologically mediated.

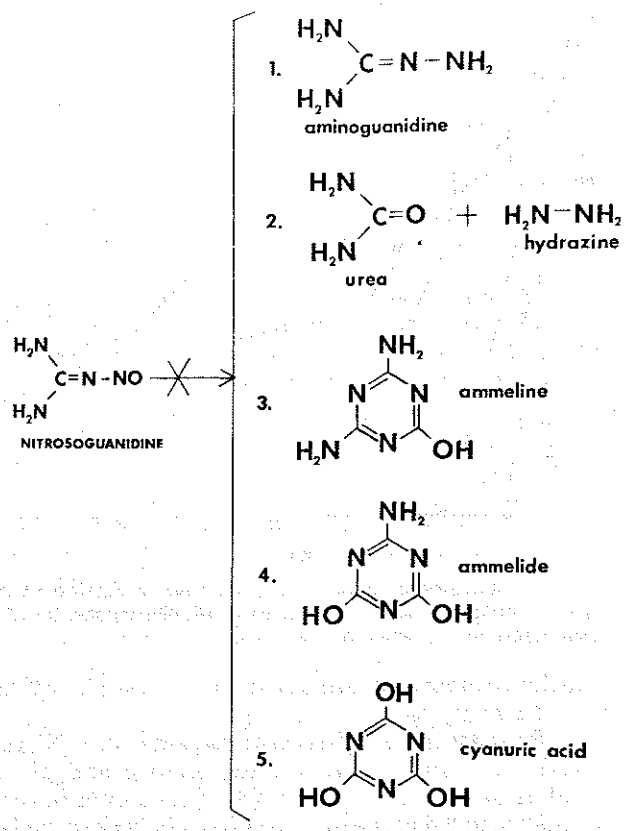


Figure 5. Products not detected during nitrosoguanidine decomposition.

Under more harsh chemical conditions, Davis and Rosenquist (*4*) reported that nitrosoguanidine reacts chemically with ammonia to give cyanamide and nitrosamide. With excess ammonia this reaction proceeds to guanidine, melamine, ammeline, ammelide, and a trace of urea. Presumably, under the milder conditions of the present biological studies, the hydroxylated analogues of melamine are not formed. Figure 5 summarizes the products not identified as nitrosoguanidine decomposition products.

Nitrosoguanidine and nitroguanidine gave negative results in the Ames screening test for mutagenicity. Nitroguanidine, however, was reported to be a carcinogen in screening tests with Chinese hamster cells for detection of chromosomal aberrations (*12*). It is important to note that *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine is a mutagen, a carcinogen, and used as DNA-alkylating agent while insufficient information is available to assess the carcinogenic hazard of nitrosoguanidine, a suspected carcinogen (*13*). Improper use of nomenclature has created confusion in the literature concerning the distinction between these two compounds.

Cyanamide is metabolized by plants to arginine through various guanidino compounds (*14*). Calcium cyanamide is used as a plant fertilizer and root stimulator (*15*) and is not carcinogenic (*16*). Cyanamide is bacteriostatic at 1000 ppm and toxic to mammals (*17*). Melamine presents a low toxicity hazard (*13*).

Results to date suggest that nitroguanidine cannot be successfully metabolized by microbial activity to completely innocuous products. Nitrosoguanidine, a nitrosamine, is the primary reduction product, and no evidence was found for further microbial action on this compound. Residual levels of nitrosoguanidine persist because of the

slow rates of the chemical reactions under these mild biological conditions. More stringent chemical conditions would presumably enhance the rate of decomposition of nitrosoguanidine and change the number and the types of products found in this study.

The demonstrated sensitivity of both nitroguanidine and nitrosoguanidine to UV light suggests this treatment as an alternative in alleviating pollution hazards associated with nitroguanidine-laden waste streams.

Acknowledgments

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Literature Cited

(1) Bissett, F. H.; Levasseur, L. A. Technical Report TR-76/47, U.S. Army Natick Res. Dev. Com. 1976.
(2) Small, M. J.; Rosenblatt, D. H. Technical Report 7404, U.S. Army Bioengineering Res. Dev. Lab. 1974.
(3) Kemula, W.; Kalinowski, M. K.; Kryogowski, T. M.; Lewandowski, J. A.; Walasek, A. J. *Bull. Acad. Pol. Sci., Chem. Ser.* **1970**, *18*, 445–461.
(4) Davis, T. L.; Rosenquist, E. N. *J. Am. Chem. Soc.* **1937**, *15*, 2112–2115.
(5) Cloak, L. H. British Patent 869 306, 1961.
(6) Diebner, R. L. U.S. Patent 3 860 594, 1975.
(7) Ames, B. N. Supplement to Method Paper, University of California, Berkeley, 1979, 1–10.
(8) Ames, B. N.; McCann, J.; Yamasaki, E. *Mut. Res.* **1975**, *31*, 347–364.
(9) Milks, J. E.; Janes, R. H. *Anal. Biochem.* **1956**, *28*, 846–849.
(10) Korn, E. D. "Methods in Enzymology IV, Purines and Pyrimidines"; Academic Press: New York, 1957; 631–632.
(11) Knappe, E.; Rohdewald, I. *Zeit. Anal. Chem.* **1966**, *223*, 174–181.
(12) Ishidate, M.; Odashima, S. *Mut. Res.* **1977**, *48*, 337–354.
(13) Sax, M. I. "Dangerous Properties of Industrial Materials"; Van Nostrand Reinhold: New York, 1975.
(14) Wvensch, A.; Amberger, A. A. *Z. Pflanzenphysiol.* **1974**, *72*, 359–366.
(15) Iwasaki, K.; Weaver, R. J. *J. Am. Soc. Hort. Sci.* **1977**, *102*, 584–587.
(16) National Cancer Institute, "Bioassay of Calcium Cyanamide for Possible Carcinogenicity"; Nat. Tech. Inf. Ser. PB-293625, 1979.
(17) Iskandarov, T. I. *Gig. Primen., Toksikol. Pestits. Klin. Otravlenii* **1970**, *8*, 337–340.

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